## REMARKS

### The Amendments

Claims 19-29 were pending, all of which have been rejected. Claims 20-22 and 25-27, are canceled herein. Claims 19 and 23 are amended. Claims 30-33 are added, and now pending.

Claim 19 is amended to recite a method to obtain a protein from a transgenic host plant, wherein step a) is now limited by the subject matters of previous claims 21 and 22 and step e) is now limited to displacing the gas phase in the reaction chamber with nitrogen and carbon dioxide. Support for this amendment is found on page 7, line 1 and paragraph 2, line 4 and page 16, line 37. Claim 19 is also amended to include a cultivation step b), a transfer of plant material step d) and protein recovery step f). Support for this amendment can be found in Examples 1 and 2, pages 16 and 17.

Claim 23 is amended to include Adh1 promoter. Support for this amendment can be found on page 10, paragraph 2.

Support for new Claims 30-33 can be found on page 7, line 6 and page 15, paragraph 2.

No new matter is introduced.

### Rejections for Indefiniteness under 35 U.S.C. § 112, Second Paragraph

Claims 19-29 are rejected as indefinite. Examiner states that the recitation of "said gas phase is rapidly displaced" in independent Claim 19 is indefinite because the metes and bounds of "rapidly" are not apparent. Claim 19 has been amended to remove the word "rapidly". Accordingly, Applicants respectfully request that the indefiniteness rejection be withdrawn.

### Rejections for Lack of Enablement under 35 U.S.C. § 112, First Paragraph

Claims 19-29 are rejected lack of enablement of the invention. Claims 20-22 and 25-27 have been canceled. The rejection of the remaining claims is traversed in parts and overcome in parts in view of amendments.

Claim 19 has been amended to recite a method of obtaining a protein from a transgenic host plant wherein the transgenic host plant comprises a protein coding gene that is functionally linked to an anaerobically inducible promoter, wherein the protein expression is induced under anaerobic conditions wherein the gas in the container for protein expression is displaced by

introducing a gaseous inductor selected from the group consisting of nitrogen and carbon dioxide into said reaction container which is completely enabled throughout the specification.

The Examiner states that the system according to the claimed invention as a whole is unpredictable. The Examiner describes the system as a whole as such; specific expression of regulatory sequences (1), + encoding the specific desired target protein (2), + the specific plant (3), + the specific tissue of the plant (4), + the environmental manipulation of the inducing conditions (5), and claims this system is unpredictable. Applicants assert that Items 1-4 describe the general process of genetic transformation of plants which was routine and predicable at the time the invention was made. The evidence for the routine genetic transformation of a wide variety of plants is evidenced in the scientific literature and numerous patents cited in the present application.

Item 5, the environmental manipulation of the inducing conditions, is clearly described and enabled throughout the specification and particularly in Examples 1 and 2. Example 1 shows that the transgenic plants were cultivated under common agricultural conditions prior to the harvest (Claim 19, step b) and how to induce the protein expression by displacing the original gas in the container with nitrogen or carbon (Claim 19, step e).

The system as a whole is further enabled throughout the specification. It is mentioned in the specification that both monocot and dicot plants can be transformed even with the same vector systems (page 6, paragraph 2). The specification provides enablement for how to construct a vector comprising a gene coding for a desired protein being linked with an inducible promoter which is active under anaerobic conditions (page 3, last paragraph through page 6, paragraph 2). Promoters which are inducible under anaerobic condition and the anaerobic inducting conditions are described and enabled on page 10, paragraph 2. Further, it was known that the function of a promoter was not dependent on the specific gene it directed.

The application contains sufficient information to enable one skilled in art to make and use the claimed invention as now amended without undue experimentation. Accordingly, Applicants respectfully request that the lack-of-enablement rejection be withdrawn.

**Rejections for Anticipation under 35 U.S.C. § 102(e)**

Claims 19-29 were rejected under 35 U.S.C.102(b) over Cerff, *et. al.* U.S. Patent 6,194,201. Claims 20-22 and 25-27 have been canceled. The rejection of the remaining claims

are traversed in parts and overcome in parts in view of amendments. Claim 19 has been amended as described in the previous section.

Cerff *et. al.* teaches a method of expressing a non-native gene under anaerobic conditions. Applicants respectfully assert that Cerff *et. al.* '201 fails to point out each and every limitation of the claimed invention as amended. The reference does not teach e) displacing the gas phase in said reaction container by introducing a gaseous inductor selected from the group consisting of nitrogen and carbon dioxide into said reaction container or g) recovering said expressed protein from said harvested transgenic host plant as taught in amended Claim 19 and throughout the specification.

The anaerobic induction of transgenic plants using the Anaerocult A system that is taught in Cerff *et. al.* '201 is fundamentally different that the displacement of the gas phase in the reaction chamber taught in the instant invention

A fundamental difference between the Anaerocult A system and the gas displacement method taught in the present invention is that the Anaerocult A system requires and contains a carbon source and a catalyst to perform the chemical reaction to obtain $CO_2$ from the oxygen present in the air and the carbon source present in the Anaerocult A material. This results in significant heat dissipation during the chemical reaction in the Anaerocult system which does not occur with the gas displacement method. Furthermore, as the container is air-tight, there is no exchange of the gas phase inside the container with the gas phase outside the container. Rather, such an exchange would make impossible obtain anaerobic conditions by the Anaerocult A system as new oxygen would continuously enter into the system. Thus, the Anaerocult A system provides a method to chemically modify a gas phase which is strictly separated from its surroundings by binding oxygen and reacting it to $CO_2$ in a closed system. This is in contrast to the method described in the instant invention, wherein the gas phase in the reaction container is physically displaced by a gaseous inductor.

Another fundamental difference between the Anaerocult A system and the gas displacement method disclosed in the present invention is that the former is slow (on the order of an hour or more) and the gas displacement method is rapid (on the order of minutes) (Imhof and Heinzer, Journal of Clinical Microbiology, July 1996, p 1646-1648 page 1647, Table 1). The difference in rate of change of the gas phase allows the Anaerocult A system to mimic the natural situation of gradual oxygen depletion over at least an hour or more which allows a

hypoxic acclimation of plants by adapting their metabolism under decreased oxygen concentration and switch to glycolysis as well as fermentation before the full anaerobiosis occurs (*e.g.* see '201 col. 2, line 36-54). On the contrary, the physical displacement by displacing the original oxygen carrying gas phase and introducing nitrogen or carbon dioxide into the container as defined in Claim 19 e) results in an immediate anoxia.

Further, Cerff *et. al.* '201 does not teach e) recovering of expressed protein from harvested transgenic host plant. The referenced patent describes merely detection of expressed protein using Northern blot analysis (column 4, line 8). Northern Blotting is a method of detecting RNA fragments that are separated by gel electrophoresis. The referenced patent does not, teach the recovery of a protein from transgenic host plant.

Therefore, failing to disclose every element of the claimed invention, the '201 patent cannot anticipate Claim 19. For at least the same reasons, the '201 patent cannot anticipate Claims 23, 24, 28, and 29, which depend from Claim 19.

Applicants respectfully request that this rejection be withdrawn.

**Rejections for Obviousness under 35 U.S.C. § 103 (a)**

Claims 19-29 have been rejected under U.S.C. 103(a) as being unpatentable over Cerff '201 in view of WO 95/005555. Claims 20-22 and 25-27 have been canceled. The rejection of the remaining claims are traversed in parts and overcome in parts in view of amendments. Claim 19 has been amended as described in the previous section.

As outlined above, Cerff '201 does not teach or suggest the displacing of air in a reaction container by introducing a gaseous inductor selected from the group consisting of nitrogen and carbon dioxide into said reaction container, nor does it teach or suggest the recovery expressed protein from a harvested transgenic host plant.

An unexpected advantage of the present invention is that there is no heat dissipation in the reaction container when using the gas displacement method, in contrast to the significant heat dissipation which occurs during the chemical modification of the gas phase in the Anaerocult A system. Thus, the chemical modification of the gas phase as described in Cerff '201 is inapplicable to large scale recovery of heat-sensitive compounds such as proteins. Applicant submit that the prior art does not render the present invention obvious. Furthermore, the prior art teaches away from the claimed invention that protein expression can be induced

and recovered upon displacing the gas in the container by introducing nitrogen or carbon dioxide into the container.

WO 95/00555 teaches a method of using a ligand binding domain (LBD) recombinase system. WO/ 95/0055 does not cure the defects of the '201 patent.

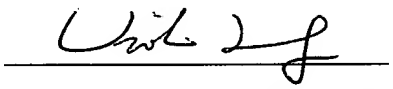
As noted in the previous section, the physical displacement by displacing the original oxygen carrying gas phase and introducing nitrogen or carbon dioxide into the container as defined in Claim 19 e) results in an immediate anoxia. It is reported in the literature at the time of the invention, that plants survive anoxia for only 4, 6, or 12 hours (Germain *et. al.* Plant Physio., 1997, 114: 167-175, attached here). Additionally, Germain *et. al.* report that under direct anoxia, no new protein synthesis was detectable in the analyzed native plants (page 171, left column lines 13-15). Therefore, one skilled in the art would not have expected that a direct anoxia-based protein production system would function in a transgenic plant. Consequently, a skilled person would not have been motivated to make a post-harvest protein production system wherein the gas phase of the reaction container is displaced from the container by introducing nitrogen or carbon dioxide into the container resulting in anoxia.

Applicants respectfully request that the obviousness rejection be withdrawn.

## CONCLUSION

The Applicants believe that the application is in good and proper condition for allowance. Early notification of allowance is earnestly solicited. The Examiner is invited to telephone the undersigned if further discussion should facilitate moving this application to allowance.

Respectfully submitted,



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Germain *et. al.* Plant Physio., 1997, 114: 167-175

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# Continuous Monitoring of Oxygen Concentrations in Several Systems for Cultivation of Anaerobic Bacteria

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**Anaerobic chambers and jars are the two conventional methods used in clinical microbiology laboratories to produce an anaerobic atmosphere. The evacuation-replacement method, the Oxoid AnaeroGen, the Merck Anaerocult A, the BBL GasPak, the BBL GasPakPlus, the Adams Scientific GasGendicator, the Difco Anaerobic, and the bioMérieux Generbox anaer systems were compared for the timed decrease in the oxygen concentration in an anaerobic jar. The experiment was repeated 10 times for each system. The oxygen concentration was measured with an oxygen analyzer series 3600 instrument (Orbisphere Laboratories, Neuchâtel-Geneva Switzerland). The BBL GasPak, the BBL GasPakPlus, the bioMérieux Generbox, the Adams Scientific GasGendicator, and the Difco Anaerobic systems contain sodium borohydride, which liberates hydrogen. The Anaerocult A system contains iron powder which binds the oxygen. The active ingredient of the AnaeroGen system is ascorbic acid. The times to reach an $O_2$ concentration of 0.5% were 8 to 15 min for the evacuation-replacement method, 26 to 41 min for the AnaeroGen system, 60 to 93 min for the Anaerocult A system, and 22 to 419 min for the sodium borohydride systems. The AnaeroGen system, the Anaerocult A system, and the evacuation-replacement method never failed to produce an anaerobic atmosphere. The sodium borohydride systems failed in 10 of 70 runs. These results suggest that the evacuation-replacement method or the Oxoid AnaeroGen system should be used to produce an anaerobic atmosphere. The Anaerocult A system showed a good reproducibility, but the length of time required to reach an appropriately low level of oxygen was too long. Because of the high failure rate, the borohydride systems cannot be recommended.**

The clinical importance of anaerobic bacteria in human infections has been well documented (5). Loesche (7) showed that strictly anaerobic bacteria are very susceptible to atmospheric oxygen. After 20 to 40 min of exposure to air, there was a 30 to 70% reduction in colony counts. The cultivation of strict anaerobes requires the rapid generation of an atmosphere with an oxygen level below 0.5% (7). This can be achieved either by the evacuation-replacement method or with a chemical system in a jar. Many chemical systems produce hydrogen and carbon dioxide from tablets of sodium borohydride, sodium bicarbonate-citric acid (Generbox anaer system; bioMérieux, Marcy-l'Etoile, France; BBL GasPak and BBL GasPakPlus systems; Becton Dickinson Microbiology Systems, Cockeysville, Md.; Anaerobic System, Difco Laboratories, Detroit, Mich.; GasGendicator system; Adams Scientific Inc., West Warwick, R.I.). The sodium borohydride-sodium bicarbonate system and the evacuation-replacement method require a palladium catalyst to generate water from hydrogen and oxygen. Some newer systems with internal generators do not require a catalyst or hydrogen to generate an anaerobic atmosphere. The Anaerocult A system (Merck, Darmstadt, Germany) contains iron powder that chemically binds oxygen. Carbon dioxide is also liberated from sodium bicarbonate and citric acid. Another anaerobic system is the AnaeroGen system (Oxoid Unipath Ltd., Basingstoke, United Kingdom), which contains ascorbic acid. The AnaeroGen system absorbs oxygen and generates a carbon dioxide concentration of between 9 and 13%. No hydrogen is produced. We compared the AnaeroGen system, the Anaerocult A system, the Difco Anaerobic System, the BBL GasPak system, the BBL GasPakPlus system, the GasGendicator System, Generbox anaer system, and the evacuation-replacement method for their abilities to decrease the oxygen concentration in an anaerobic jar.

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## MATERIALS AND METHODS

An Almore (Almore International, Inc., Portland, Oreg.) anaerobic jar (2.5 liters) was used for all anaerobic systems. The original lid was replaced with a lid of stainless steel (Fig. 1), with a socket for the oxygen sensor, a pressure sensor, and two inlets pipes. Table 1 shows the principles behind the anaerobic systems. All anaerobic systems were tested at ambient temperature (10) without plates of medium in the jar. Additionally, the AnaeroGen and the BBL GasPak systems were also tested with 10 plates of BBL brucella agar at ambient temperature and with 10 plates of BBL brucella agar at 35°C. New plates of medium were used in each experiment.

**Evacuation-replacement method.** A catalyst chamber containing 2.5 ± 0.5 g of palladium catalyst (bioMérieux) was placed in the jar. The air was evacuated with

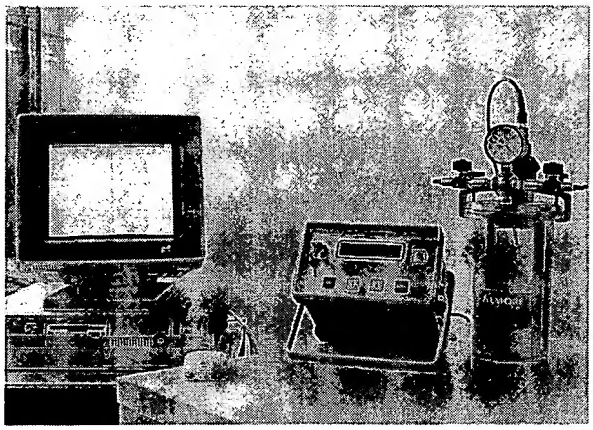

FIG. 1. Anaerobic jar with an oxygen analyzer (MOCA series 3600) connected to a personal computer.

TABLE 1. Time to reach an $O_2$ concentration of <0.5%, number of failures in 10 experiments, and principles behind the anaerobic systems

| Method or system | Presence of plates in the jar | Temp (°C) | Principle of the anaerobic system | Time (min) to reach 0.5% $O_2$ concn | | | | Failure rate[a] |
|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | Minimum | Maximum | |
| Evacuation-replacement method | No | Ambient | | 11 | 2.3 | 8 | 15 | 0/10 |
| AnaeroGen (Oxoid) | No | Ambient | Ascorbic acid | 34 | 4.0 | 26 | 41 | 0/10 |
| AnaeroGen (Oxoid) | Yes | Ambient | Ascorbic acid | 34 | 6.2 | 26 | 42 | 0/10 |
| AnaeroGen (Oxoid) | Yes | 35 | Ascorbic acid | 29 | 5.1 | 25 | 42 | 0/10 |
| Anaerocult A (Merck) | No | Ambient | Iron powder | 80 | 12.5 | 60 | 93 | 0/10 |
| Anaerobic System (Difco) | No | Ambient | Borohydride | 98 | 9 | 86 | 106 | 2/10 |
| GasGendicator (Adams Scientific) | No | Ambient | Borohydride | 93 | 73.3 | 22 | 250 | 2/10 |
| GasPak (BBL) | No | Ambient | Borohydride | 128 | 27.0 | 71 | 146 | 1/10 |
| GasPak (BBL) | Yes | Ambient | Borohydride | 96 | 24.2 | 70 | 138 | 1/10 |
| GasPak (BBL) | Yes | 35 | Borohydride | 72 | 15.1 | 46 | 85 | 1/10 |
| GasPakPlus (BBL) | No | Ambient | Borohydride | 134 | 27.8 | 71 | 146 | 1/10 |
| Generbox anaer (bioMérieux) | No | Ambient | Borohydride | 370 | 104.3 | 112 | 419 | 2/10 |

[a] Failure was considered an $O_2$ concentration at 1 h of >16%. The failure rate is indicated as number of failures/total number of tests.

a vacuum pump (Speedivac ES 35; Edwards Ltd., Crawley, United Kingdom) to $-0.7 \times 10^5$ Pa (−0.7 bar) and was replaced with a gas mixture of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen. This procedure was repeated once.

**Chemical systems.** The manufacturers' instructions for the commercial systems were strictly observed.

**Catalyst.** The palladium pellets were reactivated in a hot-air oven at 160°C for at least 1 h (3). The catalyst pellets were reactivated after each use. The Adams Scientific GasGendicator system was tested with a BBL catalyst; for all other systems the catalysts provided with the systems were used.

**Oxygen concentration.** The oxygen concentration was measured with a MOCA series 3600 analyzer, and the data were acquired by the computer program moca3600 (Orbisphere Laboratories, Neuchâtel-Geneva, Switzerland). The electrode was standardized at atmospheric pressure (measured with the built-in atmospheric pressure sensor) and the normal oxygen concentration (20.9%) (2). The oxygen concentrations and temperature were recorded every minute. The recording started 1 min after the anaerobic jar was closed and was stopped when the oxygen concentration decreased to below 0.1% $O_2$. We reported a failure of the system if the oxygen concentration did not reach 16% in 1 h. In a series of 10 tests with each system, calculations were made for 9 tests because of one failure and of 8 tests because of two failures.

## RESULTS AND DISCUSSION

In the present study seven anaerobic systems were compared for their abilities to decrease the oxygen concentration in an anaerobic jar. The carbon dioxide concentration is also important, but it was not the subject of the present study. The data are presented in Table 1 and Fig. 2. To our knowledge, this is the first investigation which compared the oxygen levels in several anaerobic systems. Previous studies have been based on

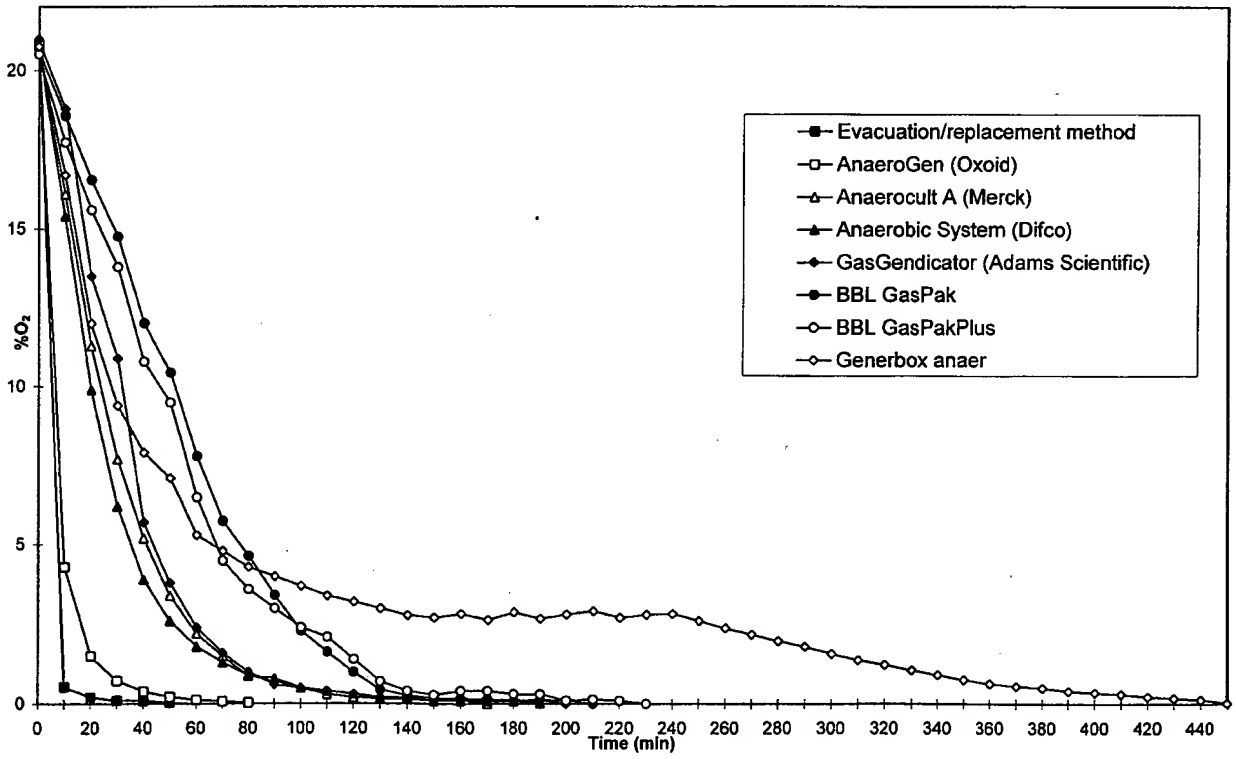


FIG. 2. Mean oxygen concentrations in the tested anaerobic systems at ambient temperature.

the growth of various anaerobic bacteria (1, 4, 6, 8, 9, 11–14) or on an atmospheric analysis of only one (10) or two (13) anaerobic systems.

Miller et al. (8) and Brazier and Hall (1) evaluated the AnaeroGen and the BBL GasPak systems for the growth of anaerobic bacteria. Miller et al. (8) reported a failure rate of 10% with the BBL GasPak system. They reported that the indicator strip was reduced faster with the AnaeroGen system than with the GasPak system. We observed no failure with the evacuation-replacement method, the AnaeroGen system, or the Anaerocult A system. A 10 to 20% failure rate was observed with the sodium borohydride systems. The BBL GasPak system also showed a 10% failure rate at 35°C.

The Anaerogen system had the advantage of requiring neither water nor a palladium catalyst. All sodium borohydride systems and the Anaerocult A system require water to start the reaction.

These results indicate that the AnaeroGen system or the evacuation-replacement method are the most rapid and reliable systems for generating an anaerobic environment. Their routine use may result in the enhanced recovery of anaerobes from clinical specimens.

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# The Role of Sugars, Hexokinase, and Sucrose Synthase in the Determination of Hypoxically Induced Tolerance to Anoxia in Tomato Roots

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**Hypoxic pretreatment of tomato (*Lycopersicon esculentum* M.) roots induced an acclimation to anoxia. Survival in the absence of oxygen was improved from 10 h to more than 36 h if external sucrose was present. The energy charge value of anoxic tissues increased during the course of hypoxic acclimation, indicating an improvement of energy metabolism. In acclimated roots ethanol was produced immediately after transfer to anoxia and little lactic acid accumulated in the tissues. In nonacclimated roots significant ethanol synthesis occurred after a 1-h lag period, during which time large amounts of lactic acid accumulated in the tissues. Several enzyme activities, including that of alcohol dehydrogenase, lactate dehydrogenase, pyruvate decarboxylase, and sucrose synthase, increased during the hypoxic pretreatment. In contrast to maize, hexokinase activities did not increase and phosphorylation of hexoses was strongly inhibited during anoxia in both kinds of tomato roots. Sucrose, but not glucose or fructose, was able to sustain glycolytic flux via the sucrose synthase pathway and allowed anoxic tolerance of acclimated roots. These results are discussed in relation to cytosolic acidosis and the ability of tomato roots to survive anoxia.**

Roots from different plants display similarly limited resistance to anoxic shock. Maize (Saglio et al., 1988; Johnson et al., 1989) and wheat (Waters et al., 1991) primary roots do not resist more than 8 to 10 h of anoxic shock. An early study by Webb and Armstrong (1981) with pea, pumpkin, and rice roots showed that apices survived only 6, 12, and 4 h of anoxia, respectively. In wheat and maize a hypoxic pretreatment has been reported to enhance survival, from 24 h for wheat (Waters et al., 1991) to more than 96 h for maize (Saglio et al., 1988; Johnson et al., 1989). Studies of the effects of hypoxic acclimation in maize root tips led to the conclusion that two main strategies are involved in improved tolerance to anoxia. One involves a better regulation of cytosolic pH (Roberts et al., 1985) by mechanisms largely independent of ATP levels (Xia et al., 1995); the other involves maintenance of glycolytic flux at a level sufficient to supply the ATP necessary to sustain cell functions (Xia et al., 1995).

The precise mechanisms involved in acclimation processes are still unknown. Induction of lactic acid excretion has been correlated with better survival of acclimated tissues (Xia and Saglio, 1992; Rivoal and Hanson, 1993), but the importance of excretion in cytosolic pH regulation is doubtful (Xia and Roberts, 1996), and whether lactic acid accumulation alone accounts for cytosolic acidosis is still a matter of debate (Roberts et al., 1984a; Saint-Gès et al., 1991). Among the proteins induced during hypoxic acclimation, only HKs have been demonstrated to contribute to the survival of acclimated maize root tips (Bouny and Saglio, 1996) by allowing the maintenance of a sustained glycolytic rate. Induction of ADH (Andrews et al., 1994) does not appear to be related to anoxic tolerance. The low activity found for PDC in aerobic tissues and its substantial induction in hypoxia suggested that its activity might be limiting in nonacclimated tissues (Waters et al., 1991). However, PDC is also strongly activated by the low cytosolic pH (Davies et al., 1974) usually found in nonacclimated anoxic tissues (Xia et al., 1995; Bouny and Saglio, 1996), and hypoxic induction of PDC may not, therefore, be necessary to allow anoxic tolerance.

Most metabolic studies on hypoxic acclimation have been with monocotyledons (for review, see Ricard et al., 1994). An exception is the study by Rivoal and Hanson (1994) on lactate glycolysis in transgenic tomato roots overexpressing barley LDH. In an effort to understand the changes that give rise to improved stress resistance and to see whether the mechanisms implicated in cereals can be generalized to dicotyledons, we studied the effect of hypoxic pretreatment on the survival of tomato roots. In particular, we sought to determine whether the accumulation of lactic acid in the tissues could account for differential anoxic tolerance of tomato and whether particular enzyme activities were limiting and induced by hypoxia, with special attention paid to HK and SS as possible controlling steps of glycolytic flux in anoxia, depending on sugar supply.

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Abbreviations: ADH, alcohol dehydrogenase; AEC, adenylate energy charge; ANP, anaerobic proteins; cfu, colony-forming units; dGlc, 2-deoxyglucose; FK, fructokinase; GK, glucokinase; HK, hexokinase; HPT, hypoxically pretreated; INV, invertase; LDH, lactate dehydrogenase; NHPT, not hypoxically pretreated; PDC, pyruvate decarboxylase; SS, Suc synthase.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Tomato (*Lycopersicon esculentum* M., var UC 82b, Versailles) seeds were germinated and grown for 20 d in potting soil before transfer to 20-L plastic tanks for hydroponic growth under controlled conditions (12-h day at 25°C/12-h night at 20°C). The nutrient solution, containing nutrients at a concentration four times greater than that described in Saglio and Pradet (1980), was continuously sparged with air. After 7 d a 20-h hypoxic pretreatment was applied by bubbling the nutrient solution with 3% oxygen in nitrogen. Shoots were always maintained in air. Plants submitted to hypoxic pretreatment are henceforth referred to as HPT and the aerobic controls as NHPT, respectively. Anoxic treatment was imposed on roots excised 10 cm from the apex.

Since the plant material used was nonsterile, measures were taken to reduce microbial contamination. Roots were carefully washed in deionized water when plants were transferred to hydroponic cultivation, and again washed five times in sterile water before experimental use. This measure was reported to decrease by 100-fold the number of cfu on the roots of hydroponically grown *Limonium* spp. (Rivoal and Hanson, 1993) to levels ($10^6$ cfu $g^{-1}$ fresh weight) that did not interfere with the use of radiotracers in studies of root metabolism. In comparison with *Limonium* spp. cultivated hydroponically for long periods, less microflora contamination was expected on young plants grown hydroponically for only 1 week. Control experiments confirmed that washing reduced the bacterial counts found on the terminal 10-cm region of the youngest roots to less than $10^5$ cfu $g^{-1}$ fresh weight. Bacterial counts were determined by plating root brei on Luria broth agar plates (1.5% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1.0% [w/v] NaCl) and incubating for 4 d at 25°C. In addition, the bacteriostat cefotaxime (250 $\mu$g $mL^{-1}$), added at the beginning of short-term-radiotracer and in vitro-metabolite experiments, was found to maintain the bacterial counts at a constant level for the duration of the experiment. Cefotaxime has been tested for use in cell cultures and is certified by the manufacturer (Duchefa, Haarlem, The Netherlands) to have no cytotoxic effects. It is routinely used to eliminate *Agrobacterium* from transformed plant tissues.

### Survival and Adenine Nucleotide Determination

Root anoxic tolerance is most often evaluated in terms of capacity to elongate after return to air. However, in this study anoxic treatment was imposed on detached roots, for which elongation was difficult to measure. We therefore evaluated anoxic tolerance as the maintenance of the sum of adenine nucleotides (ATP + ADP + AMP) at a level compatible with that of living tissue, i.e. greater than 10% of the aerobic level. This method has proven to be reliably correlated to viability (Bouny and Saglio, 1996).

For anoxic treatments, 50 mg of root segments was placed in polyethylene tubes containing 2 mL of MS/4 medium (Murashige and Skoog diluted 4-fold, pH 5.8) and 250 $\mu$g $mL^{-1}$ cefotaxime supplemented with 100 mM Suc or Glc and fitted with caps. Hypodermic needles inserted through the rubber caps were used to gas the solution and headspace, one needle bubbling gas into the solution, the other allowing gas to exit from the tube. After the desired time of incubation the solution was forced out of each tube by inverting the gas connections, and the tube was placed in liquid nitrogen to quickly freeze the roots in the absence of contact with air. The frozen tubes were stored at −20°C and nucleotides were extracted for ATP, ADP, and AMP bioluminescence estimation using procedures published previously (Saglio et al., 1988).

### Ethanol and Lactate Accumulation

Root segments (200 mg fresh weight) were placed in penicillin vials containing 2 mL of MS/4 medium and 250 $\mu$g $mL^{-1}$ cefotaxime supplemented or not with 100 mM Suc, Glc, or Fru. The vials were flushed for 5 min with pure nitrogen, sealed, and placed on a rotary shaker at 200 rpm. At the indicated times, the nutrient medium was removed and conserved at −20°C in sealed tubes until assayed. The root segments were rapidly rinsed five times with 5 mL of cold water by vacuum depression, blotted, reweighed, and frozen in liquid nitrogen. Frozen tissues were ground and extracted with perchloric acid according to the method of Bouny and Saglio (1996). Lactic acid was determined enzymatically in the incubation medium and tissue extracts, and ethanol was assayed in the medium alone as in Saglio et al. (1980).

### Enzyme Activities

Root samples (200 mg) were homogenized in a volume (in microliters) corresponding to twice the fresh weight of tissue (in milligrams) and desalted exactly as described in Bouny and Saglio (1996). Soluble proteins were estimated on HPT and NHPT root samples homogenized in the same buffer devoid of BSA, according to the method of Bradford (1976), with BSA as a reference. For both types of roots, 200 mg fresh weight corresponded to 1 mg of soluble protein; this ratio did not change during anoxic treatment.

Activities of GK, FK, LDH, ADH, and PDC were assayed by spectrophotometry at 25°C and pH 7.5, as described in Bouny and Saglio (1996). Activities of neutral INV and SS were measured successively by spectrophotometry at pH 7.0, whereas acid INV was assayed at pH 4.8, essentially according to the method of Nguyen-Quoc et al. (1990).

### Soluble Sugar Determinations

A known amount of sorbitol (absent in the tissues) was added as an internal standard to the frozen, excised root segments prepared as described for lactic acid and ethanol accumulation. The samples were extracted successively every 15 min with 1 mL of hot ethanol:water (80:20, 50:50, 0:100, v/v, at 80°C). The extracts were dried under a vacuum and solubilized in 500 $\mu$L of water.

For sugar determinations, 400 $\mu$L of extract was purified on tandem ion-exchange resins consisting of 0.4 mEq of resin (AG 1-X8, Bio-Rad) in the carbonate form and 0.5 mEq of resin (Dowex-50W, Sigma) in the $H^+$ form. The purified

soluble sugars were analyzed by HPLC (Aminex HPX-87C column from Bio-Rad with water as an eluant at 3.6 mL $h^{-1}$ at 75°C) with refractometric detection.

### In Vivo Labeling of Proteins and Two-Dimensional Gel Electrophoresis

Roots of HPT or NHPT plants were carefully washed. Four lots of 20 excised root tips (1.5 cm in length; 80 mg fresh weight) were placed in polyethylene tubes containing 1.5 mL of MS/4 medium supplemented with 100 mM Suc and 250 $\mu$g $mL^{-1}$ cefotaxime. Different gas treatments were applied by bubbling NHPT roots for 6 h with 50% oxygen in nitrogen (aerobic control), 3% oxygen in nitrogen (hypoxic treatment), 100% nitrogen (anoxic treatment), and HPT roots with 100% nitrogen for the hypoxic pretreatment followed by anoxic treatment. After the first 2 h of each treatment, 500 $\mu$Ci of Tran$^{35}$S-label (1150 Ci $mmol^{-1}$, $^{35}$S-Met/Cys 70/30; ICN) was added. At the end of the 4-h labeling period, root tips were rinsed five times with sterile water and frozen in liquid nitrogen. Bacteriological controls carried out on an aliquot of the incubation medium at the end of the experiment showed less than $10^4$ cfu $g^{-1}$ fresh weight.

Roots conserved at −80°C were ground with a glass potter in twice their fresh weight of a buffer containing 50 mM Tris-HCl (pH 6.8), 2% (v/v) β-mercaptoethanol, 2% (w/v) SDS, and 10% (w/v) glycerol. The brei was heated for 3 min at 80°C, then centrifuged for 15 min in an Eppendorf centrifuge. To the supernatant was added 2 volumes of buffer containing 9.0 M urea, 2% ampholines, 2% β-mercaptoethanol, and 8% (w/v) Nonidet P-40. For gel analysis, samples were deposited on tube gels for IEF in the first dimension as described by O'Farrell (1975). The second dimension was a discontinuous SDS slab gel system, with 15 and 5% (w/v) acrylamide:bisacrylamide (36.5:1) in the separating and stacking gel, respectively.

### Analysis of Glc-P and of dGlc Phosphorylation Rate

Glc-6-P in neutralized perchloric extracts of 200 mg of root segments (as described above for lactic acid) were assayed enzymatically in 1-mL reaction mixtures according to the method of Burrell et al. (1994). In recovery experiments carried out in parallel, known amounts of Glc-6-P, comparable to those found in the tissues, were added to the frozen samples before extraction. Three independent assays gave recoveries ranging from 92 to 100%.

The rate of in vivo phosphorylation was assayed using a labeled, nonmetabolizable analog of Glc, dGlc (2-deoxy-D-[U-$^{14}$C]Glc, 11.8 GBq $mmol^{-1}$; Dupont/NEN). One-hundred-milligram samples of HPT and NHPT root segments were treated as described in Bouny and Saglio (1996), except that 0.2 mM labeled dGlc (275 MBq $mmol^{-1}$, 55 kBq $mL^{-1}$ final concentration) was present in the anoxic medium. In the aerated solution, the specific radioactivity of the 0.2 mM dGlc solution was one-third of that used in anoxia. The amount of dGlc-P produced in 30 min was estimated by the difference in radioactivity before and after mixing 400 $\mu$L of neutralized labeled perchloric extract with 0.4 mEq (300 $\mu$L) of $HCO_3^-$ anionic resin as described in Bouny and Saglio (1996).

## RESULTS

### Effect of Hypoxic Pretreatment on Anoxic Tolerance

Figure 1 shows that in the presence of 100 mM Suc a hypoxic pretreatment of 20 h improved the anoxic tolerance of tomato roots from less than 12 h to more than 36 h. In the presence of Glc instead of Suc, HPT roots did not survive significantly longer than NHPT roots, in spite of better maintenance of the nucleotide level during the first 5 h of anoxic incubation. Data obtained on 100 mM Fru or in the absence of added sugar in the medium did not differ significantly from those obtained with Glc (not shown). Consequently, the hypoxic pretreatment had a positive effect on survival of excised tomato roots only when Suc was present in the incubation medium.

In anoxic conditions HPT roots had a higher AEC than NHPT roots, depending on the duration of HPT (Table I). Starting from values close to 0.3, AEC measured after 45 min of anoxia increased rapidly during the first 3 h, and then very slowly to values close to 0.6 after 22 h of hypoxic treatment. On the basis of this result, a period of 20 h was chosen for hypoxic acclimation of tomato roots. Higher AEC values are an indication of improved energy metabolism (Saglio et al., 1980; Raymond et al., 1983). Since fermentation is the principal catabolic pathway during anoxia, we studied the effects of the hypoxic pretreatment on ethanol and lactate production.

### Effects of a Hypoxic Pretreatment on Ethanol and Lactate Synthesis

After transfer of HPT tomato roots to anoxia in medium supplemented with Suc, ethanol was immediately pro-

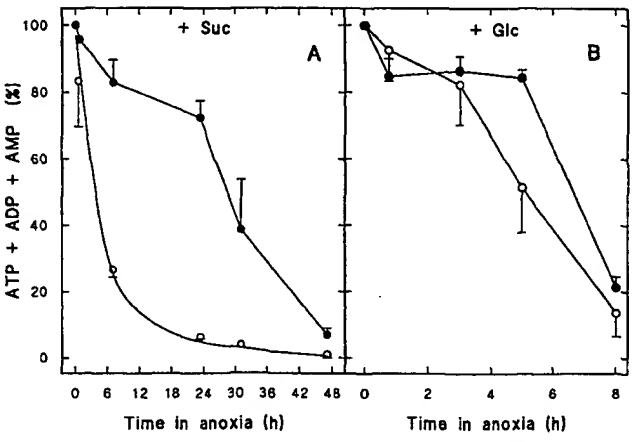


**Figure 1.** Total adenine nucleotides in excised tomato roots with time under anoxia. Data are shown for HPT (●) and NHPT (○) roots supplemented with 100 mM Suc (A) or 100 mM Glc (B). Points are the means ± SD of three independent determinations. Adenine nucleotide concentrations are given as the percentage of the time zero value in air for each treatment. One hundred percent corresponded to 101 or 101 and 125 or 137 pmol adenine nucleotides $mg^{-1}$ fresh weight for HPT and NHPT on Suc or Glc, respectively.

**Table I.** *Variations of adenine nucleotides and AEC under anoxia as a function of the hypoxic (3% oxygen in nitrogen) pretreatment time*

Values were obtained after transfer of root segments to anoxia for 45 min. Data are the means ± SD of three independent repetitions.

| Nucleotide | Time of Hypoxic Pretreatment | | |
|---|---|---|---|
| | 0 h | 3 h | 22 h |
| | *pmol mg$^{-1}$ fresh wt* | | |
| ATP | 13 ± 1 | 23 ± 3 | 27 ± 2 |
| ADP | 30 ± 2 | 33 ± 2 | 30 ± 1 |
| AMP | 59 ± 6 | 36 ± 2 | 19 ± 1 |
| AEC[a] | 0.28 | 0.42 | 0.56 |

[a] AEC = ([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]).

duced at a rate of about 5 nmol mg$^{-1}$ fresh weight h$^{-1}$ (Fig. 2). In NHPT roots, ethanol was produced after a 1-h lag period and at a lower rate (2.9 nmol mg$^{-1}$ fresh weight h$^{-1}$). The substitution of Glc for Suc strongly modified the time course of ethanol production in both kinds of roots (Fig. 2). NHPT roots produced only a very small amount of ethanol during the first hour after transfer to anoxia. In HPT roots the rate of ethanol was significant (2.7 nmol mg$^{-1}$ fresh weight h$^{-1}$) during 3 h and then stopped almost completely. In the absence of sugar in the incubation medium the time course of ethanol production was similar but lower than that obtained with Glc. Data with 100 mM Fru did not differ significantly from those obtained on Glc (not shown).

Figure 3 shows the distribution of lactic acid between the tissues and the surrounding medium. HPT roots accumulated only small amounts of lactic acid in the tissues regardless of the sugar present in the medium (Fig. 3, A and B). The accumulation occurred mainly during the 1st h after transfer to nitrogen and then declined slowly, with a concomitant accumulation in the medium. In contrast, NHPT roots supplemented with 100 mM Suc accumulated large amounts of lactic acid in their tissues (more than five times that in HPT roots). Eighty percent of this accumulation occurred during the 1st h of anoxia, which corresponds to the lag period for ethanol production. The total lactic acid produced (tissue plus medium) by NHPT roots on 100 mM Suc was 3-fold that produced by HPT roots after 7 h of anoxia. In contrast, when Glc was substituted for Suc, accumulation of lactic acid in the tissue and in the medium was only slightly higher (less than 1.5-fold) in NHPT than in HPT roots, and the time course was almost identical.

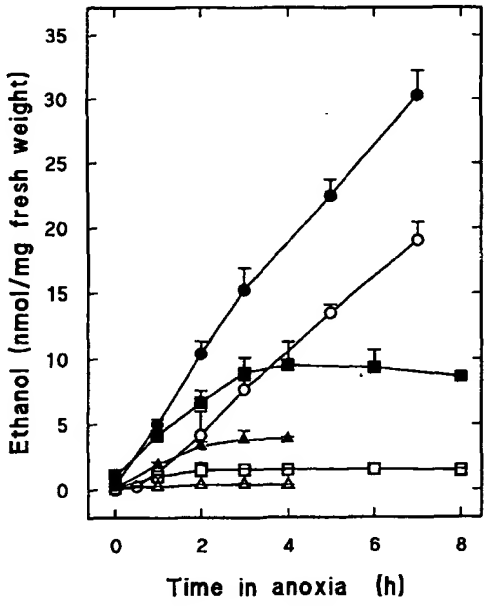


**Figure 2.** Time course of ethanol production by excised tomato roots. Data are shown for HPT roots, supplemented with 100 mM Suc (●) or Glc (■) or in the absence of added sugars (▲), and NHPT roots, supplemented with 100 mM Suc (○) or Glc (□) or in the absence of added sugars (△). Each point is the mean ± SD of three independent determinations.

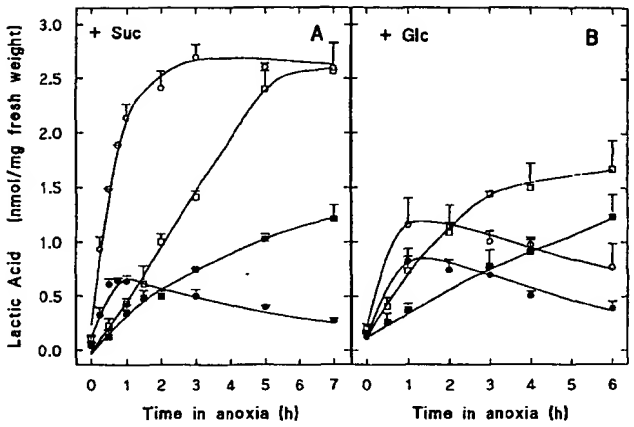


**Figure 3.** Time course of lactic acid production in the presence of 100 mM Suc (A) or 100 mM Glc (B). Data are shown in the tissues for HPT (●) or NHPT (○) roots and in the incubation medium for HPT (■) and NHPT (□) roots. Each point is the mean ± SD of three independent determinations.

## Activities of Glycolytic and Fermentative Enzymes

A 20-h hypoxic pretreatment of intact tomato root systems induced an increase in the in vitro activities of all of the fermentative enzymes tested (Table II). This increase was 5-fold for ADH and 3-fold for LDH and PDC. Among the enzymes involved in the first steps of sugar utilization, only SS activity increased 3-fold, whereas neutral and acid INV activities decreased. In contrast to maize (Bouny and Saglio, 1996), HK (including GK and FK) activities did not increase following the hypoxic pretreatment; its activities remained the lowest among the enzymes tested, especially after the hypoxic pretreatment. To ascertain that the low values found were not the result of losses during extractions and assays, recovery experiments were carried out. In three independent trials in which amounts of pure commercial enzyme (bakers' yeast) comparable to that found in root tissues were added to the frozen samples before grinding, the recoveries were in the range of 79 to 86%, with a 10% loss at the desalting step.

**Table II.** *In vitro enzyme activities of NHPT and HPT tomato roots*

Immediately after 20 h of pretreatment, excised roots were homogenized for enzyme activity measurements. Data are means ± SD of three independent repetitions.

| Enzyme | NHPT Roots | HPT Roots |
|---|---|---|
| | *pmol product min$^{-1}$ mg$^{-1}$ fresh wt* | |
| GK | 70 ± 5 | 75 ± 10 |
| FK | 220 ± 45 | 215 ± 25 |
| SS[a] | 525 ± 70 | 1620 ± 320 |
| INV (neutral) | 700 ± 5 | 590 ± 95 |
| INV (acid) | 3625 ± 85 | 2085 ± 220 |
| LDH | 85 ± 10 | 290 ± 60 |
| ADH | 405 ± 45 | 2265 ± 325 |
| PDC | 290 ± 20 | 795 ± 40 |

[a] Results are expressed in pmol Fru min$^{-1}$ mg$^{-1}$ fresh wt.

### Anaerobic Protein Synthesis in Tomato Roots

In maize and rice increased activities of a number of glycolytic and fermentative enzymes in response to anoxia are the result of the induction of anaerobic peptides subsequent to gene activation (Ricard et al., 1994). A typical anaerobic protein profile in tomato roots would be an indication that such mechanisms are also operative in tomato. Roots excised from NHPT and HPT plants were therefore labeled with a mixture of $^{35}$S-Met and Cys for the final 4 h of a 6-h treatment under various gaseous environments, as described in "Materials and Methods." Figure 4 shows the two-dimensional profile of proteins synthesized during 4 h under aerobic conditions, during hypoxic acclimation, and during anoxia after hypoxic acclimation. In the absence of hypoxic acclimation, proteins were not detectably labeled during a 4-h anoxic incubation (data not shown). However, a hypoxic pretreatment clearly allows the labeling of a number of proteins. Comparison of the proteins labeled under anoxia with those labeled under hypoxia reveals several striking differences. First, the profile is much simpler under anoxia, with only three major spots. Second, several proteins are induced only during hypoxia, with molecular masses of about 92, 65, and 50 kD. From molecular mass considerations and induction characteristics, it is tempting to speculate that these may include SS (92.4-kD subunit molecular mass reported for tomato SS by Wang et al. [1993]) and PDC (62- and 64-kD subunit masses reported for rice PDC by Rivoal et al. [1990]). It is also noteworthy that the protein profile is strikingly similar in complexity to that of maize primary roots labeled under anoxia (Sachs et al., 1980) and very different from the simple profile of soybean (Russell et al., 1990).

### Changes in Soluble Sugars

Soluble sugars were assayed in tomato root tissues during the course of anoxic incubation in the presence or absence of added sugars in the medium (Table III). In the absence of sugars, Suc was dominant at the time of excision, followed by Fru and only trace amounts of Glc. Concentrations of Suc in HPT roots were twice those found in NHPT roots. When incubated on Suc or Glc, large amounts of Suc and Glc accumulated in excised roots during the short incubation in normoxia (15 min) needed for preparation before transfer to $N_2$. Suc accumulated about 10 times faster than Glc. Suc was exhausted after 2 or 4 h in NHPT or HPT roots, respectively, regardless of the presence or absence of Glc in the incubation medium. In roots supplemented with Suc, the pool of Suc remained very high during the 4-h experiment, with a small decrease in HPT roots. The levels of Glc and Fru did not decrease significantly in any of the situations studied and the level of Glc even increased during incubation on Glc.

### Changes in Sugar Phosphate Contents

Glc-6-P was assayed in excised NHPT and HPT tomato roots (Table IV) during incubation in the presence of 100 mM Glc. The amount of Glc-6-P was 1.5-fold higher in HPT than in NHPT roots. One hour after transfer to anoxia the pool of Glc-6-P had dropped dramatically in both kinds of roots, to represent only 7 and 20% of the initial pool after 4 h in NHPT and HPT roots, respectively. When tissues were returned to air the Glc-6-P concentration increased in 1 h to more than 50% of its initial value.

The phosphorylation activity of HKs, assayed in vivo using dGlc, is also presented in Table IV. The percentage of dGlc phosphorylated in 30 min was close to 50% in both HPT and NHPT roots in air and decreased markedly after only 1 h in both types of roots, particularly in NHPT. The rate of dGlc phosphorylation increased when tissues were returned to air after 4 h in anoxia.

## DISCUSSION

The results presented here show that a 20-h hypoxic pretreatment of tomato roots induced most of the traits observed in maize root tips after a similar treatment (for review, see Ricard et al., 1994). The apparent survival of excised tomato roots in anoxia, based on adenine nucleotide levels, was significantly improved from less than 10 h to more than 36 h by the hypoxic pretreatment, provided Suc was present in the incubation medium. In contrast to maize root tips (Bouny and Saglio, 1996), Glc or Fru did not allow expression of anoxia tolerance in tomato roots (Fig. 1). Only Suc allowed this expression in correlation with its ability to maintain a sustained glycolytic rate (Fig. 2). As in maize root tips (Xia and Saglio, 1992), the AEC value of anoxic tissues increased with longer hypoxic pretreatments, indicating the induction of a more efficient energy metabolism, in acclimated tomato roots. As in NHPT maize (Xia and Saglio, 1992) and other plant tissues (Davies et al., 1974; Roberts et al., 1984b), there was a lag period in NHPT tomato roots before significant ethanol production and the large concomitant accumulation of lactic acid in the tissues. This contrasts with HPT roots, in which there was no lag period for ethanol production and only a slight accumulation of lactic acid in the tissues. As in maize and many other plant tissues (for review, see Ricard et al., 1994), a number of enzyme activities were induced during the hypoxic pretreatment, including SS and the fermentative enzymes ADH, LDH, and PDC.

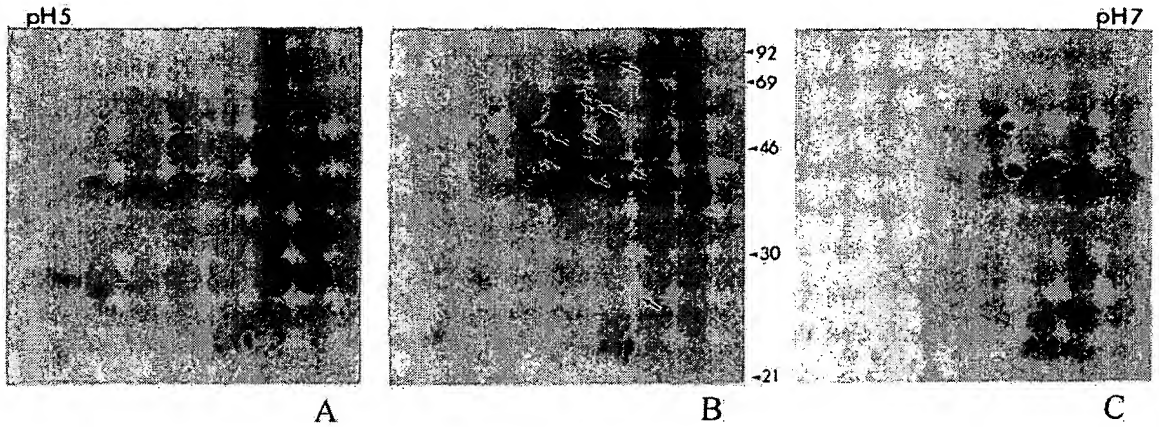


**Figure 4.** Changes in protein synthesis in tomato roots in response to hypoxia and to anoxia after hypoxic pretreatment. Proteins synthesized in air (A), during hypoxic pretreatment (B), and during anoxia following a hypoxic pretreatment (C) were analyzed by two-dimensional IEF/SDS-PAGE. The specific radioactivities of the protein samples analyzed were 48,000 (A), 22,000 (B), and 2,200 (C) cpm $mg^{-1}$ protein, respectively. The numbers to the right of gel B indicate the position of the molecular mass markers (kD). The arrows show the major proteins induced during hypoxic pretreatment.

Tomato roots differed from maize roots in several aspects. In contrast to maize (Xia and Saglio, 1992), the higher AEC was correlated with a higher glycolytic flux using Suc not only after long-term incubation in anoxia, but also immediately after transfer to nitrogen. This rate, estimated as the sum of ethanol plus lactate (accounting for more than 80% of the total fermentative flux, data not shown), was close to 6 and 2.5 nmol $mg^{-1}$ fresh weight $h^{-1}$ for HPT and NHPT roots, respectively, during the 1st h after transfer to nitrogen, and about 3.8 and 3.0 nmol $mg^{-1}$ fresh weight $h^{-1}$, respectively, after 3 h. The 37% decline of the total glycolytic flux in HPT roots was mainly the consequence of the arrest of lactate synthesis, which was not balanced by a rise in ethanol production. This situation contrasted with NHPT roots, in which the arrest of lactate synthesis was almost entirely balanced by the increase in ethanol synthesis, allowing the glycolytic rate on Suc to remain almost stable, and only 15% lower than in HPT roots after 3 h of anoxic incubation. The decline of glycolytic flux in HPT roots supplemented with Glc or Fru corresponds precisely to the depletion of Suc in the tissues. Similarly, the very low fermentation of NHPT roots supplemented or not with Glc or Fru can be correlated with the small concentration of Suc present in the tissues. These findings imply that at least part of the Suc escapes cleavage by the cell wall INV and enters the cell intact, where it is metabolized exclusively via the SS pathway. This allows the bypass of the HK step, which was almost inoperative

**Table III.** *Soluble sugar content of tomato root segments during incubation in anoxia*

NHPT and HPT excised roots were incubated in the presence of 100 mM Suc or 100 mM Glc, or in the absence of added sugars. Each value is the mean ± SD of three independent determinations.

| Time | Sugars | Sugars Supplied in the Medium | | | | | |
|---|---|---|---|---|---|---|---|
| | | 100 mM Suc | | 100 mM Glc | | No sugars | |
| | | NHPT | HPT | NHPT | HPT | NHPT | HPT |
| *h* | | *nmol Eq Glc $mg^{-1}$ fresh wt* | | | | | |
| | Suc | 16.6 ± 0.72 | 20.3 ± 0.46 | 1.3 ± 0.05 | 3.8 ± 0.17 | 1.0 ± 0.03 | 2.2 ± 0.21 |
| 0[a] | Glc | 2.7 ± 0.40 | 2.8 ± 0.13 | 1.9 ± 0.18 | 3.9 ± 0.31 | Traces | Traces |
| | Fru | 4.2 ± 0.31 | 4.8 ± 0.16 | 0.3 ± 0.05 | 1.5 ± 0.11 | 0.5 ± 0.01 | 0.6 ± 0.10 |
| | Suc | 18.0 ± 0.40 | 20.4 ± 1.48 | 0.8 ± 0.09 | 2.6 ± 0.54 | 0.6 ± 0.08 | 1.1 ± 0.06 |
| 1 | Glc | 2.6 ± 0.60 | 3.2 ± 0.30 | 1.1 ± 0.38 | 3.2 ± 0.34 | Traces | Traces |
| | Fru | 5.0 ± 0.88 | 5.8 ± 0.05 | 0.3 ± 0.06 | 1.0 ± 0.03 | 0.4 ± 0.02 | 0.4 ± 0.01 |
| | Suc | 18.2 ± 0.99 | 18.7 ± 0.77 | 0.3 ± 0.02 | 1.8 ± 0.58 | 0.4 ± 0.06 | 0.6 ± 0.05 |
| 2 | Glc | 2.7 ± 0.63 | 2.9 ± 0.22 | 3.3 ± 0.10 | 3.3 ± 0.29 | Traces | Traces |
| | Fru | 3.9 ± 0.31 | 5.0 ± 0.40 | 0.4 ± 0.01 | 0.9 ± 0.16 | 0.3 ± 0.03 | 0.4 ± 0.01 |
| | Suc | 18.4 ± 0.45 | 17.4 ± 0.77 | 0.3 ± 0.08 | 0.7 ± 0.06 | 0.4 ± 0.13 | 0.3 ± 0.04 |
| 3 | Glc | 2.3 ± 0.14 | 2.5 ± 0.12 | 4.4 ± 0.35 | 3.2 ± 0.47 | Traces | Traces |
| | Fru | 3.6 ± 0.34 | 5.2 ± 0.27 | 0.3 ± 0.01 | 0.8 ± 0.06 | 0.2 ± 0.06 | 0.3 ± 0.04 |
| | Suc | 19.2 ± 1.25 | 15.5 ± 0.39 | 0.3 ± 0.06 | 0.4 ± 0.07 | 0.3 ± 0.06 | 0.2 ± 0.06 |
| 4 | Glc | 2.0 ± 0.15 | 2.2 ± 0.12 | 5.8 ± 1.00 | 4.2 ± 0.23 | Traces | Traces |
| | Fru | 3.3 ± 0.14 | 4.6 ± 0.29 | 0.4 ± 0.10 | 0.6 ± 0.08 | 0.1 ± 0.02 | 0.2 ± 0.02 |

[a] Zero time represents 15 min of aerobic incubation in the presence or absence of sugars.

**Table IV.** *Glc-6-P content and rate of dGlc phosphorylation in excised NHPT and HPT tomato roots during incubation in anoxia*

Control in air corresponds to the end of the pretreatment period of the intact plants. The rates of dGlc phosphorylation are expressed as a percentage of dGlc-6-P in the total dGlc absorbed after 30 min of incubation in anoxia. Each data point is the mean ± SD of three independent determinations.

| Treatment | Glc-6-P | | Total dGlc Absorbed | | Rate of Phosphorylation | |
|---|---|---|---|---|---|---|
| | NHPT | HPT | NHPT | HPT | NHPT | HPT |
| | *pmol $mg^{-1}$ fresh wt* | | *pmol $mg^{-1}$ fresh wt* | | *% of total dGlc absorbed* | |
| Control in air | 530 ± 15 | 785 ± 29 | 175 ± 46 | 151 ± 29 | 47 ± 4 | 46 ± 4 |
| 1 h in Nitrogen | 55 ± 23 | 180 ± 30 | 26 ± 1 | 36 ± 3 | 13 ± 3 | 26 ± 2 |
| 4 h in Nitrogen | 35 ± 11 | 140 ± 12 | 23 ± 2 | 23 ± 2 | 1 ± 3 | 23 ± 3 |
| 4 h in Nitrogen + 1 h in air | 300 ± 35 | 420 ± 21 | 62 ± 8 | 125 ± 8 | 28 ± 3 | 54 ± 8 |

during anoxia in NHPT tomato roots and strongly reduced in HTP roots, as discussed below.

In vitro activities of GK and FK are low and, at least for GK, not far from the minimum required to account for the glycolytic flux measured in HPT roots (50 pmol of Glc $mg^{-1}$ fresh weight $min^{-1}$, compared with 75 and 215 pmol $mg^{-1}$ fresh weight $min^{-1}$ for GK and FK, respectively). In addition, and this was a major difference with maize root tips, HK (including GK and FK) activities did not increase during the hypoxic pretreatment. As suggested by Renz and Stitt (1993) and as demonstrated in maize root tips (Bouny and Saglio, 1996), HKs are strongly inhibited by the low pH and low ATP concentrations of anoxic tissues. That this inhibition occurred in the tomato roots is shown by the rapid decrease in the Glc-6-P pool (Table IV), even in the presence of high concentrations of Glc and Fru in the tissues (Table III), and also by the low rate of in vivo dGlc phosphorylation. This decrease was not the result of proteolytic degradation because these activities increased when tissues were returned to air after 4 h in anoxia. The higher Glc-6-P concentration in HPT roots even after 4 h of anoxic treatment might be the result of some remaining activity of the SS pathway, leading to the production of Glc-1-P in equilibrium with Glc-6-P through UDPGlc pyrophosphorylase activity or, more probably, to a less inhibitory environment (higher pH and higher ATP levels) for HK activities in accordance with the fact that there was still some phosphorylation capacity in these tissues (Table IV).

The results obtained with tomato roots differ from those published by Rivoal and Hanson (1994) on tomato root clones in that the latter are apparently capable of metabolizing Glc under anoxia. This may be because of differences in HK activities (or inducibility) in transformed root clones or in different tomato varieties. However, in both types of material, HPT results in more efficient excretion of lactate into the medium.

The extent of cytosolic acidosis in plant tissues has been widely shown to correlate with their capacity to survive in the absence of oxygen (for review, see Ricard et al., 1994). We have not measured cytosolic pH directly. However, it is interesting to evaluate the impact of lactic acid accumulation on the cytosolic pH of tomato roots. On a fresh weight basis and according to Roberts et al. (1992), the maximum lactic acid accumulation occurring in 60 min in NHPT tomato roots corresponded to 2.5 $\mu$Eq $H^+$ $g^{-1}$ tissue. This value is very close to the 3 $\mu$Eq $H^+$ $g^{-1}$ tissue reported for maximum accumulation of lactic acid in maize root tips, which occurs in 20 min in this fast-metabolizing tissue (Roberts et al., 1992). Assuming a buffering capacity similar to maize root tissues of 14 $\mu$Eq $H^+$ $g^{-1}$ (Roberts et al., 1981), the production of lactic acid alone should account for a decrease in cytosolic pH of about 0.4 unit. This value is probably largely underestimated because the proportion of cytosol to vacuole in the tomato root segments used in this study was certainly much lower than in maize root tips, as suggested by the soluble protein content, which was 6-fold higher in maize root tips per unit fresh weight. Nevertheless, this simple calculation suggests that the massive accumulation of lactic acid in anoxic NHPT tomato roots may play a significant role in their sensitivity to this stress.

### Anaerobic Protein Synthesis in Tomato Roots

Anaerobiosis has long been known to affect protein synthesis, classically repressing the synthesis of preexisting proteins and inducing that of a limited set of new proteins. This shift in the pattern of protein synthesis has been observed in the roots of both monocotyledons (maize, Sachs et al. [1980]; rice, Bertani et al. [1981] and Mocquot et al. [1981]; Echinochloa, Mujer et al. [1993]) and dicotyledons (Arabidopsis, Dolferus et al. [1985]; soybean, Russell et al. [1990]). The anaerobic protein profile of soybean is the simplest. Only four spots, of which the most intensely labeled comigrated with ADH, were resolved on two-dimensional native/SDS-PAGE (Russell et al., 1990), leading to speculations that the high sensitivity of soybean to flooding stress might be related to limited ANP induction. In much of the work discussed above, a period of hypoxia before the actual anoxic treatment was neither excluded nor controlled. It has also been noted that a gradual decrease in oxygen concentration corresponds more closely to physiological reality than anoxic shock. We have chosen to mimic the process of gradual anoxia by a controlled pretreatment under hypoxia. The protein profile obtained under such conditions (Fig. 4C) can be assimilated to what have been called ANPs in the literature, which are probably in fact synthesized under anoxia only by acclimated tissues. This is supported by the fact that no incorporation of label into protein could be detected during anoxic shock when care was taken to exclude a period of hypoxia before the application of anoxia, both in tomato roots and in maize primary roots (V. Germain, B. Ricard, P. Raymond, and P.H. Saglio, unpublished data; Andrews et al., 1994).

Our work shows a correlation between the increased capacity for protein synthesis and improved tolerance in HPT tomato roots during anoxia. However, most of the enzymes involved in energy metabolism during anoxia are present under aerobic conditions and it has proved difficult to show that induction is necessary for the expression of tolerance. The only exceptions are HKs in maize (Bouny and Saglio, 1996). It is worthwhile to note that the situation in tomato roots lends support to the necessity for ANP induction. HKs are not induced by acclimation and the existing levels do not permit the use of Glc and Fru as substrates for glycolysis during anoxia. Thus, HPT roots are unable to express anoxic tolerance on Glc and Fru. Improved tolerance of HPT tomato roots in the presence of Suc alone implies the possibility of bypassing the HK step via SS. Whether the observed induction of SS is necessary is unknown.

## CONCLUSION

The results presented here confirm that HKs are markedly inhibited in anoxic tomato roots, as was already reported in maize (Bouny and Saglio, 1996). This inhibition was such that only Suc was able to fuel glycolysis via the SS pathway. The significance of a 3-fold increase of SS in vitro activity in HPT roots (Table II) is not clear because the activity in NHPT roots was already far above the minimum required to account for the maximum glycolytic flux. Transformed tomato plants with enhanced HK activities are predicted to show improved sugar utilization and glycolysis in anoxia. The use of such plants should help to determine to what extent the maintenance of a high glycolytic rate can by itself improve tolerance to anoxia of HPT and also of NHPT tomato roots. These studies are currently in progress.

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